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An Important Role of VEGF-C in Promoting Lymphedema Development

Epameinondas Gousopoulos¹, Steven T. Proulx¹, Samia B. Bachmann¹, Lothar C. Dieterich¹, Jeannette Scholl¹, Sinem Karaman^{1,2}, Roberta Bianchi¹ and Michael Detmar¹

Secondary lymphedema is a common complication after cancer treatment, but the pathomechanisms underlying the disease remain unclear. Using a mouse tail lymphedema model, we found an increase in local and systemic levels of the lymphangiogenic factor vascular endothelial growth factor (VEGF)-C and identified CD68⁺ macrophages as a cellular source. Surprisingly, overexpression of VEGF-C in a transgenic mouse model led to aggravation of lymphedema with increased immune cell infiltration and vascular leakage compared with wild-type littermates. Conversely, blockage of VEGF-C by overexpression of soluble VEGF receptor-3 reduced edema development, diminishing inflammation and blood vascular leakage. Similar findings were obtained in a hind limb lymph node excision lymphedema model. Flow cytometry analyses and immunofluorescence stainings in lymphedematous tissue showed that VEGF receptor-3 expression was restricted to lymphatic endothelial cells. Our data suggest that endogenous VEGF-C causes blood vascular leakage and fluid influx into the tissue, thus actively contributing to edema formation. These data may provide the basis for future clinical therapeutic approaches.

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INTRODUCTION

Lymphedema constitutes the cardinal manifestation of lymphatic malfunction, characterized by lymphatic stasis, profound inflammation, and fibroadipose tissue accumulation (Rockson, 2000). It commonly occurs upon lymphatic injury due to surgical cancer treatment or radiotherapy, with breast cancer survivors representing most patients affected (Cormier et al., 2010; Rockson and Rivera, 2008). Despite the large number of patients developing lymphedema, no curative treatment exists so far, and the knowledge about the pathomechanisms that govern the development of lymphedema is rather limited and incomplete.

There is increasing evidence suggesting that lymphedema development is a multistep process, where lymphatic injury is the trigger of a sequence of pathological consequences (Rockson, 2008). Lymphedema develops in only a fraction of cancer survivors (up to 30%) (Warren et al., 2007) and mostly in a delayed fashion, indicating that secondary events might be requisite for the development of the disease. Lymphedema may even appear after seemingly minor injuries of the

lymphatic vasculature (e.g., lymph node biopsy), indicating that a simple “stopcock” mechanism, due to obstruction of lymphatic fluid transport, fails to explain many clinical aspects of lymphedema pathology (Stanton et al., 2009).

Edema, inflammation, and fibroadipose tissue deposition represent the best characterized features of lymphedema pathophysiology, but the contribution of other cellular components to the development of the disease has not been extensively studied. Stanton and colleagues (Stanton et al., 2009) reported that blood capillary angiogenesis occurs in the skin of swollen lymphedematous arms, whereas lymphatic flow and lymphatic capillary width were increased in the contralateral arm in patients with breast cancer-related lymphedema (BCRL). Moreover, global abnormalities in lymphatic function were detected in patients who developed BCRL, as were higher pumping pressures in women destined to develop BCRL. These findings indicate factors that predispose patients to lymphedema development or systemic changes after surgery or other treatments (Bains et al., 2015; Cintolesi et al., 2016).

Recently, a systemic increase in vascular endothelial growth factor (VEGF)-C levels was reported in BCRL patients, associated with increased forearm capillary filtration capacity (Jensen et al., 2015). Even though adenoviral delivery of VEGF-C had beneficial effects in lymph node engraftment and formation of new lymphatic vessels in animal models (Tammela et al., 2007), the relevance of VEGF-C for lymphedema pathogenesis has remained unknown (Visuri et al., 2015). In particular, it remains unclear whether endogenously produced VEGF-C plays a detrimental or a beneficial role in the process of lymphedema development.

In this study, we used an established mouse tail model of secondary surgical lymphedema (Gousopoulos et al., 2016b) and genetic mouse models for VEGF-C gain and loss of function to investigate the potential role of VEGF-C in the

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Abbreviations: BCRL, breast cancer-related lymphedema; K, keratin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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pathophysiology of the disease. We found increased levels of VEGF-C both locally and systemically during lymphedema development. Overexpression of VEGF-C in the skin of keratin (K) 14–VEGF-C transgenic mice surprisingly aggravated lymphedema development, leading to increased immune cell infiltration and increased blood vascular leakage. Conversely, neutralization of VEGF-C/D in K14-sVEGF receptor (VEGFR)-3-Ig transgenic mice led to the opposite results. The morphological and histopathological findings were further confirmed in gain- and loss-of-function studies in the hind limb mouse lymphedema model. We found that VEGFR3 expression was confined to lymphatic endothelial cells in lymphedema, indicating that VEGF-C promotes vascular leakage through VEGFR2. Together, these data point to a critical, active role of VEGF-C in promoting the pathogenesis of lymphedema.

RESULTS

VEGF-C increases locally and systemically during lymphedema development and is produced by CD68⁺ macrophages

To investigate the dynamics of expression of VEGFs during the course of lymphedema development, we surgically induced secondary lymphedema in the tails of wild-type mice and analyzed the mRNA expression of VEGF-A, VEGF-C, and VEGF-D in tail skin lysates at different time points. A significant increase in VEGF-C expression was detected 2 weeks ($P < 0.05$) and 6 weeks after surgery ($P < 0.01$) (Figure 1a). There was a significant up-regulation of VEGF-C after 6 weeks ($P < 0.05$). In contrast, VEGF-A expression levels were decreased 2 weeks after surgery ($P < 0.01$) (Figure 1a).

We next investigated whether this increase in VEGF-C/D mRNA would result in elevated systemic levels of VEGF-C or VEGF-D during the course of lymphedema. VEGF-C and VEGF-D protein levels were measured by ELISA in mouse serum, and a systemic increase of VEGF-C was detected 6 weeks after surgery ($P < 0.001$), whereas the levels of VEGF-D remained largely unchanged during the course of lymphedema development (Figure 1b).

Because increased levels of VEGF-C were detected both locally and systemically upon lymphedema development in our mouse model, we next aimed to determine the cellular source of VEGF-C. To this end, we subjected VEGF-C-LacZ reporter mice to surgical lymphedema induction and evaluated X-Gal staining in the lymphedematous tail tissue 2 weeks later. Using this approach, we identified CD68⁺ macrophages to be the major source of VEGF-C expression in this model (Figure 1c). Furthermore, we isolated CD11b⁺/F4/80⁺ and CD11b⁺/F4/80[−] cells from control (unoperated) and lymphedematous mouse tails. Quantitative PCR analysis of VEGF-C and VEGF-D showed that VEGF-C expression was significantly increased in CD11b⁺/F4/80⁺ macrophages ($P < 0.05$) upon induction of lymphedema. Similarly, VEGF-D expression appeared to be increased in CD11b⁺/F4/80⁺ macrophages as well, without reaching statistical significance (Figure 1d).

Skin-specific VEGF-C overexpression exacerbates lymphedema

Because lymphedema induced local and systemic increases of VEGF-C, we next evaluated whether VEGF-C might play

a beneficial or detrimental role in lymphedema. K14–VEGF-C mice, producing human VEGF-C in epidermal keratinocytes under control of the K14 promoter, and their wild-type littermates had surgery and were monitored for 2 weeks. Tail volume measurements indicated increased edema in the transgenic versus wild-type mice already at 1 week after surgery ($P < 0.01$), which remained significantly elevated 2 weeks after the operation as well ($P < 0.01$) (Figure 2a). Evaluation of the change of tail volume showed a 66.07% greater volume increase in the K14–VEGF-C transgenic mice than in the wild-type mice at 2 weeks after the operation, leading to significantly more edematous tails (Figure 2a, 2b).

To further validate our findings, we used an additional acute lymphedema model, in which edema was induced by removing the popliteal lymph node (Frueh et al., 2016). Paw thickness measurements showed significantly increased edema in K14–VEGF-C transgenic mice ($P < 0.05$, $P < 0.001$) at two timepoints after surgery (see Supplementary Figure S1a, S1b online).

Lymphangiogenesis and inflammation are characteristic features of lymphedema development (Rutkowski et al., 2006). We found that in tail sections of K14–VEGF-C mice that did not have surgery, a larger fraction of the tissue was covered by LYVE-1⁺ lymphatic vessels than in wild-type mice ($P < 0.01$), whereas no changes were detected in the area covered by Meca32⁺ blood vessels (Figure 2c). Two weeks after lymphedema induction, there was a 2- to 3-fold increase of lymphatic vessel coverage compared with untreated controls in both groups, with no major differences between transgenic and wild-type mice. Blood vessel coverage was slightly increased as well after 2 weeks in both groups (Figure 2c). In the mouse hind limb lymphedema model, increased lymphatic vessel area coverage was observed in K14–VEGF-C transgenic mice after lymphedema induction, whereas the blood vascular coverage remained unchanged (see Supplementary Figure S1c, S1d). Because CD68⁺ macrophages were identified as producers of VEGF-C during lymphedema development, we next analyzed the CD68⁺ cell infiltrate. No significant differences in the CD68-positive tissue area were found between wild-type and transgenic mice, even though a strong trend toward a higher macrophage density was observed in the hind limb model (Figure 2d, and see Supplementary Figure S2a, S2b online).

VEGF-C overexpression results in increased blood vascular leakage and altered immune cell infiltration

The increased edema formation observed in K14–VEGF-C mice might be due to changes in the inflammatory cell infiltrate and/or to increased blood vascular leakage. To evaluate the immune cell infiltrate, flow cytometry analysis was performed assessing the major immune populations within the lymphedematous tissue. At 2 weeks after surgery, there was a significant increase of CD45⁺ ($P < 0.01$), CD11b⁺ ($P < 0.05$), and CD11c⁺/F4/80⁺ cells ($P < 0.05$) compared with the wild-type mice (Figure 3a). Similarly, immunohistological analysis showed an increased immune cell infiltration in K14–VEGF-C transgenic mice in the hind limb model (see Supplementary Figure S2a, S2b).

We next investigated the effects of increased VEGF-C levels on lymphatic vascular transport function and blood

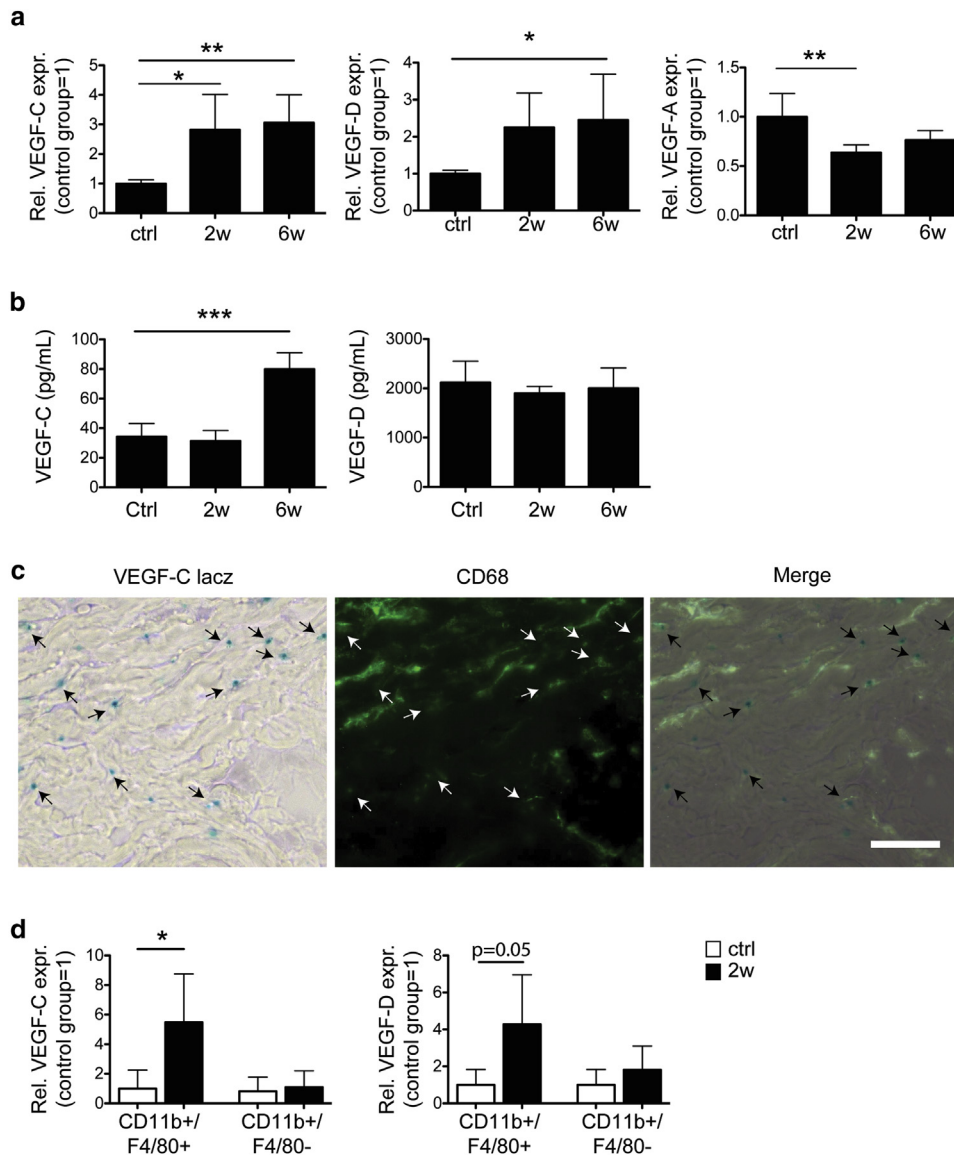


Figure 1. Local and systemic increase of VEGF-C during lymphedema development. (a) Expression levels of VEGF-C mRNA in tail skin significantly increased 2 weeks after surgery and remained elevated 6 weeks after surgery. VEGF-D mRNA expression was significantly increased after 6 weeks. VEGF-A levels were decreased 2 weeks after surgery. (b) ELISA of mouse serum showed increased systemic levels of VEGF-C at 6 weeks after surgery, but VEGF-D levels remained unchanged ($n = 5$, one-way analysis of variance with Dunnett post hoc multiple comparison test). (c) Double staining for X-gal (VEGF-C-LacZ) and CD68 2 weeks after surgery indicated that CD68⁺ macrophages constitute a major source of VEGF-C during lymphedema development. (d) Increased VEGF-C mRNA expression was identified only on isolated CD11b⁺/F4/80⁺ cells 2 weeks after surgery but not on CD11b⁺/F4/80⁻ cells. Scale bar = 50 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Ctrl., control; expr., expression; Rel., relative; VEGF, vascular endothelial growth factor; w, week.

vascular leakage by noninvasive dynamic near-infrared imaging. To examine the lymphatic vascular transport function 2 weeks after surgery, a PEGylated near-infrared dye (20kDa PEG-IRDye800), known to be taken up selectively by the lymphatic vasculature (Proulx et al., 2013b), was slowly perfused into the tip of the tail, and its transport to the edge of the excised area was monitored. Quantification of the dye 1.5 cm distal to the surgical excision area showed decreased transport of the dye in K14-VEGF-C transgenic mice ($P < 0.05$), suggesting impaired lymphatic vascular transport function after exacerbated lymphedema development (see [Supplementary Figure S3a](#) online).

Blood vascular leakage was studied distal to the surgical excision margin 1 day after the operation and before development of measurable edema, to evaluate the contribution of vessel leakage to edema formation. At this time point only increased VEGF-C and VEGF-D expression was noted, whereas VEGF-A expression remained unchanged (see [Supplementary Figure S3b–d](#)). After intravenous injection of a 20-kDa PEG-IRDye800 near-infrared tracer (Proulx et al.,

2013a), transgenic mice exhibited a 2.44-fold higher leakage rate compared with the wild-type mice ($P < 0.05$), as determined by the increase of extravasated tracer signal in the tissue (Figure 3b). This indicates that VEGF-C overexpression increases blood vessel leakiness, thus leading to increased edema formation.

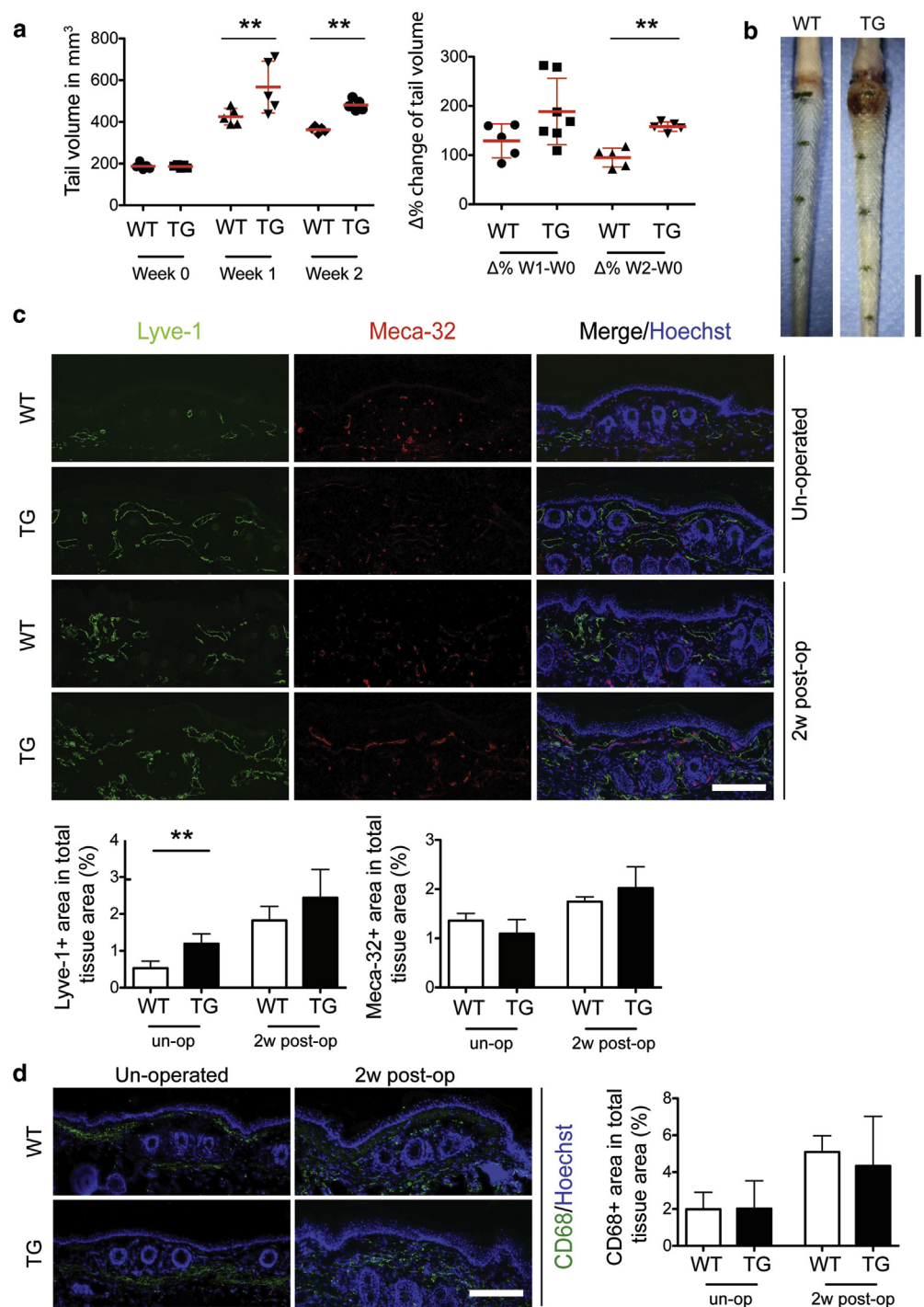
Previously, VEGF-C has been implicated in the regulation of blood pressure, which could potentially affect vascular leakage indirectly (Machnik et al., 2009). However, both systolic and diastolic blood pressures were not different between the two groups, indicating that blood pressure changes are not involved in the observed vascular leakage (see [Supplementary Figure S4a](#) online).

Reduced lymphedema development in K14-sVEGFR3-Ig transgenic mice

Because overexpression of VEGF-C aggravated lymphedema development, we next investigated whether blockade of VEGF-C might reduce lymphedema. To this end, we studied K14-sVEGFR3-Ig mice that express a soluble form of

Figure 2. Overexpression of VEGF-C exacerbates lymphedema development.

(a) Evaluation of tail volume (left) and volume change (right) showed significantly increased edema formation in K14-VEGF-C transgenic (TG) mice compared with their wild-type (WT) littermates at 2 weeks after the operation. (b) Representative pictures of WT and K14-VEGF-C TG mouse tails at 2 weeks after surgery. Scale bar = 1 cm. (c) TG mice without surgery (un-op) had an expanded lymphatic network (LYVE-1 staining, green) compared with WT mice, without major differences in blood vessels (Meca32 staining, red). Hoechst nuclear staining is in blue. At 2 weeks after induction of lymphedema, lymphatic vessels were further expanded in both groups, but no major differences between WT and TG mice were found regarding lymphatic and blood vessel coverage. (d) Staining for CD68⁺ macrophages did not show major changes between WT and VEGF-C overexpressing mice at the examined time points (n = 5–7 per group, one-way analysis of variance with Tukey post hoc multiple comparison test or two-way analysis of variance with Bonferroni post hoc test was used to compare means of three or more groups. ***P* < 0.01). Scale bars = 200 μm. post-op, after surgery; VEGF, vascular endothelial growth factor.



VEGFR3 under control of the K14 promoter, thereby scavenging the VEGFR3 ligands VEGF-C and VEGF-D in the skin. K14-sVEGFR3-Ig mice that did not have surgery exhibited a mild primary lymphedema in the tail skin (not statistically significant) (Figure 4a) and a more pronounced edema in the paw (*P* < 0.001) (see Supplementary Figure S5a online). At 1 and 2 weeks after surgery, the tail volume was comparably increased in wild-type and transgenic mice (Figure 4a). Because the K14-sVEGFR3-Ig mice exhibited a higher baseline tail volume, we calculated the volume change after surgery and found a significantly lower

increase in tail volume in the transgenic mice both 1 week (*P* < 0.01) and 2 weeks after surgery (*P* < 0.05) (Figure 4a, 4b). We further examined these observations in the mouse hind limb lymphedema model. Paw thickness measurements showed comparable changes in the wild-type and transgenic mice upon lymph node removal. Given the increased baseline thickness of the transgenic mice, the percentage thickness change was significantly lower in the transgenic group (*P* < 0.05, *P* < 0.001), further supporting the results obtained in the mouse tail model (see Supplementary Figure S5a, S5b).

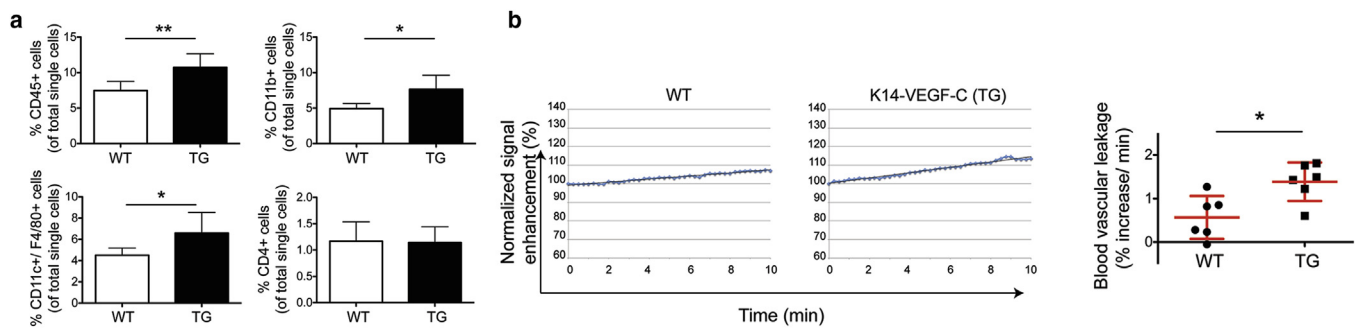


Figure 3. VEGF-C overexpression led to increased immune cell infiltration and increased blood vascular leakage. (a) Flow cytometry analysis was performed to evaluate the immune cell populations in wild-type (WT) and K14-VEGF-C transgenic (TG) mice at 2 weeks after lymphedema induction. Increased percentages of CD45⁺, CD11b⁺, and CD11c⁺/F4/80⁺ cells were noted. (b) Evaluation of the blood vascular leakage rate distal to the surgery site 1 day after the operation, before the development of measurable edema. There was a significantly increased leakage rate of the intravenously injected 20-kDa PEG-IRDye800 near-infrared tracer into the tail tissue of VEGF-C–overexpressing mice. n = 6–8; Student *t* test, **P* < 0.05. VEGF, vascular endothelial growth factor.

We next evaluated the response of lymphatic and blood vessels to VEGF-C neutralization. Untreated K14-sVEGFR3 transgenic mice had a decreased tissue area covered by LYVE-1⁺ lymphatic vessels (*P* < 0.05) and by Meca32⁺ blood vessels (*P* < 0.01) (Figure 4c). At 2 weeks after lymphedema induction, transgenic mice showed no signs of vascular expansion, resulting in significantly lower tissue coverage by lymphatic vessels (*P* < 0.01) and blood vessels (*P* < 0.05) compared with wild-type mice (Figure 4c). In the hind limb lymphedema model, lymphatic coverage was also reduced in K14-sVEGFR3-Ig mice, but no changes in the blood vascular coverage were observed (see Supplementary Figure S5c, S5d). This observation might be related to site-specific differences of the skin. Quantification of the area covered by CD68⁺ macrophages showed a comparable area in untreated mice, whereas transgenic mice had a decreased infiltration by CD68⁺ cells (*P* < 0.05) 2 weeks after surgery (Figure 4d). Similar findings were obtained in the hind limb lymphedema model (*P* < 0.01) (see Supplementary Figure S6a, S6b online).

Decreased immune cell infiltration and decreased blood vascular leakage in K14-sVEGFR3-Ig mice

Based on the reduced infiltration by CD68⁺ cells observed into the lymphedematous tail of K14-sVEGFR3-Ig transgenic mice 2 weeks after surgery, we next performed flow cytometry analyses to evaluate the immune cell infiltration in more detail. We found a trend toward decreased numbers of CD45⁺, CD11b⁺, and CD11c⁺/F4/80⁺ cells 2 weeks after surgery in K14-sVEGFR3-Ig transgenic mice (Figure 5a) and significantly reduced numbers of CD4⁺ cells (*P* < 0.05) (Figure 5a). CD4⁺ cells have previously been shown to aggravate lymphedema formation (Gousopoulos et al., 2016a; Zampell et al., 2012b).

Evaluation of blood vascular leakage 1 day after surgery showed a significantly decreased leakage rate of the intravenously injected near-infrared tracer into the tail tissue of K14-sVEGFR3-Ig mice compared with wild-type controls (*P* < 0.01) (Figure 5b).

VEGFR3 expression is restricted to lymphatic endothelial cells in lymphedematous skin

Because overexpression of VEGF-C aggravated lymphedema, whereas overexpression of the soluble form of VEGFR3 reduced lymphedema development and also decreased the

infiltration of CD68⁺ macrophages and CD4⁺ T cells, we sought to identify the cell populations in tail skin that express VEGFR3 and might thus respond to VEGF-C. To this end, we used VEGFR3-Cre-tdTomato reporter mice that express the fluorescent protein tdTomato in cells where the VEGFR3 promoter was active. VEGFR3-Cre-tdTomato mice had surgery, and the expression of VEGFR3 was evaluated by flow cytometry analysis on lymphatic and blood vascular endothelial cells and on myeloid (CD11b⁺) and T cells (CD3⁺) cells 2 weeks after surgery. tdTomato fluorescence was only detectable in lymphatic endothelial cells (CD31⁺/podoplanin⁺) but not in blood vascular endothelial cells (CD31⁺/podoplanin[−]) or immune cells (CD45⁺/CD11b⁺ and CD45⁺/CD3⁺) (Figure 6a, 6b). Furthermore, immunofluorescence double-stainings for VEGFR3 and LYVE-1, Meca-32, or CD68 showed that VEGFR3 was exclusively present on LYVE-1⁺ lymphatic vessels but not on Meca-32⁺ blood vessels or CD68⁺ macrophages (Figure 6c). Because VEGF-C is known to bind to and activate VEGFR2 in addition to VEGFR3, these data suggest that VEGF-C may regulate blood vessel leakage directly by activating VEGFR2 on blood vessel endothelial cells.

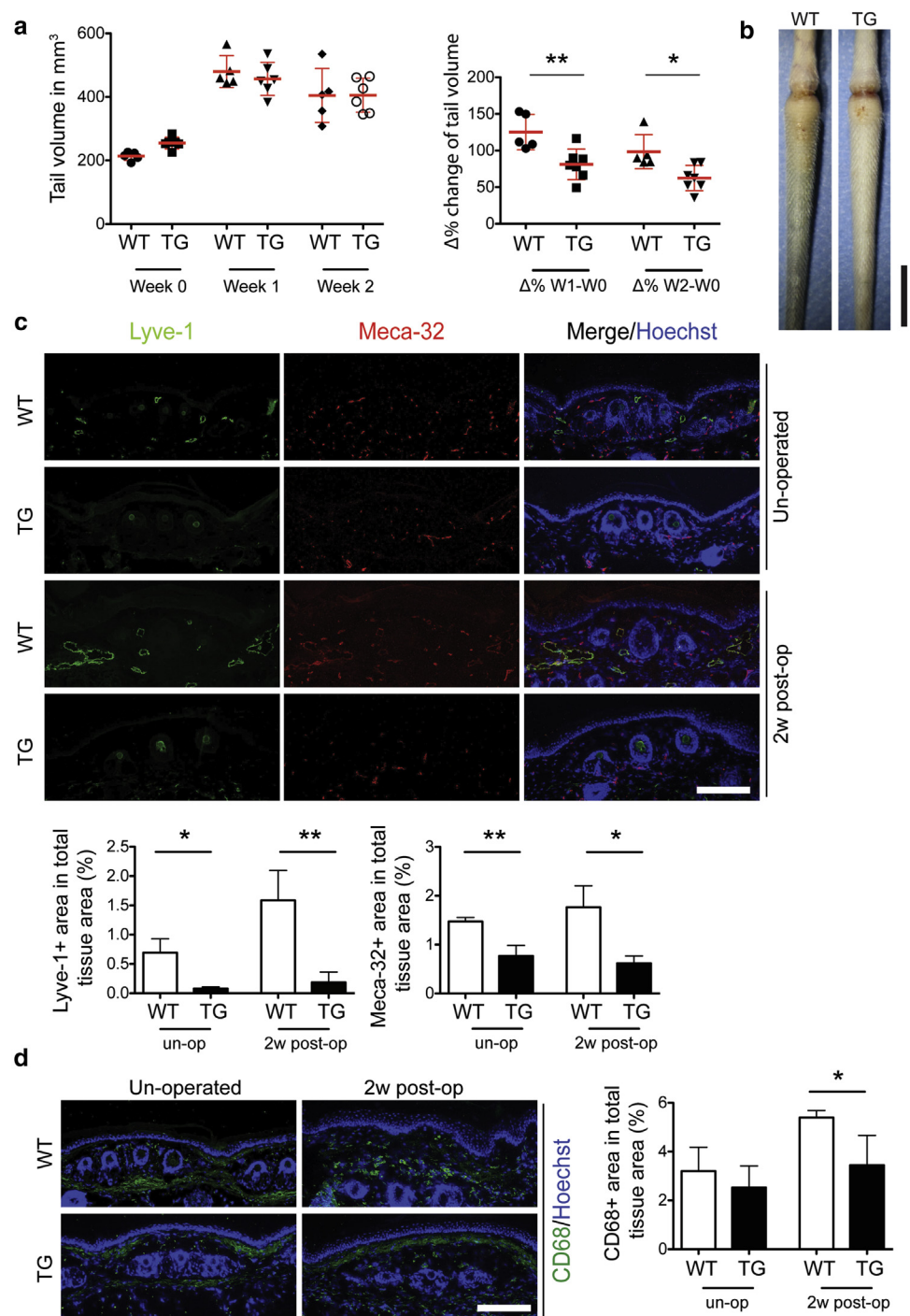
DISCUSSION

Lymphedema develops in up to 30% of breast cancer survivors after surgery and/or radiotherapy (Warren et al., 2007), with lymphatic injury considered to represent the initiator of a sequence of events that finally leads to the development of the disease. However, the consecutive steps that lead to chronic lymphedema, characterized by profound inflammation and fibroadipose tissue deposition, and the potential participation of other cellular and molecular players in the pathogenesis remain unclear.

A key finding of this study was that VEGF-C promoted blood vascular leakage in experimental, surgically induced lymphedema. VEGF-C expression in the lymphedematous tissue was up-regulated already within 2 weeks after surgery, in contrast to VEGF-A, arguing against a major role of VEGF-A in lymphedema formation. These data are in line with a recent RNA sequencing study of lymphedematous tissue (Gousopoulos et al., 2016a). Circulating VEGF-C protein levels were elevated at 6 weeks after surgery in mice, and the clinical relevance of these findings is confirmed by the

Figure 4. Reduced lymphedema development in K14-sVEGFR3-Ig transgenic mice. (a) Evaluation of tail volume (left) and volume change (right) showed increased tail volume in the K14-sVEGFR3-Ig transgenic mice without surgery and a lower percentage increase of tail volume at 1 and 2 weeks after surgery. (b)

Representative pictures of mouse tails of wild-type (WT) and transgenic (TG) mice at 2 weeks after surgery. Scale bar = 1 cm. (c) Expression of VEGFR3s led to decreased lymphatic (LYVE-1, green) and blood vessel (Meca32, red) density in the tail skin of transgenic mice both before and after lymphedema induction. Blue: Hoechst nuclear staining. (d) Whereas CD68⁺ macrophage levels were comparable in mice without surgery (un-op) of both genotypes, TG mice had a decreased CD68⁺ cell infiltration 2 weeks after lymphedema surgery. n = 5–7 per group, one-way analysis of variance with Tukey post hoc multiple comparison test or two-way analysis of variance with Bonferroni post hoc test was used to compare means of three or more groups. **P* < 0.05, ***P* < 0.01. Scale bars = 200 μ m (c and d). post-op, after surgery; VEGFR, vascular endothelial growth factor receptor.



elevated VEGF-C levels recently reported in serum samples of BCRL patients (Jensen et al., 2015). Elevated systemic levels of the potent lymphangiogenic factor VEGF-C provides a molecular explanation for the observed increased lymph drainage rate in the contralateral hand of BCRL patients and for the increased lymphatic capillary width in the contralateral forearm of these patients (Stanton et al., 2009).

VEGF-C is a major lymphangiogenic factor that enhances proliferation, migration, and survival of lymphatic endothelial cells (Tammela et al., 2005). Previously, it has been found that overexpression of either VEGF-C or VEGF-D in

experimental mouse models promotes sprouting lymphangiogenesis and lymphatic vascular enlargement, and indeed, profound enlargement of lymphatic vessels and active proliferation of lymphatic endothelial cells have also been reported in experimental models of lymphedema (Gousopoulos et al., 2016b; Rutkowski et al., 2006; Zampell et al., 2012b). Under certain experimental conditions, VEGF-C and VEGF-D overexpression may also induce angiogenesis in experimental animal models (Cao et al., 2004; Rissanen et al., 2003; Saaristo et al., 2002a; Witzenbichler et al., 1998). This activity is thought to be mediated by the

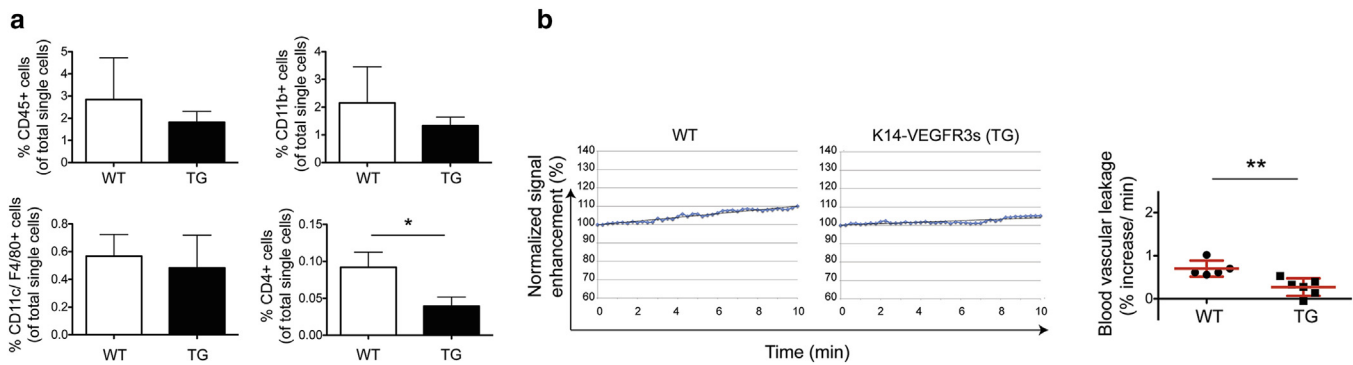


Figure 5. VEGF-C neutralization decreases immune cell infiltrate and blood vascular leakage. (a) Flow cytometry analysis was performed to evaluate the immune cell infiltration upon lymphedema induction between wild-type and transgenic mice and indicated a trend toward lessened CD45⁺ and CD11b⁺ cells and significantly reduced numbers of CD4⁺ cells. (b) The effect of sVEGFR3 on blood vascular leakage was evaluated, and leakage was found to be significantly reduced in the transgenic mice 1 day after surgery. n = 6–8; Student *t* test, **P* < 0.05, ***P* < 0.01. TG, transgenic; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WT, wild type.

proteolytically processed mature forms of VEGF-C and VEGF-D that have been reported to bind to the VEGFR2 expressed on blood vessels and to increase angiogenesis and blood flow (Anisimov et al., 2009). In this context, the recently observed increased capillary filtration rate in the affected (Jensen et al., 2015) and contralateral forearms (Stanton et al., 2009) of BCRL patients, as well as the increased lymphatic pumping in the upper limbs of patients who later develop lymphedema (Cintolesi et al., 2016), might be explained by the elevated circulating levels of VEGF-C.

Macrophages are a natural source of VEGF-C (Harvey and Gordon, 2012), and this population has previously been reported to strongly increase upon induction of experimental lymphedema (Gousopoulos et al., 2016b; Ogata et al., 2016; Zampell et al., 2012b). This is in agreement with our finding that CD68⁺ macrophages infiltrated lymphedematous tissue and were the major producers of VEGF-C, as indicated by our studies in VEGF-C lacZ reporter mice. Furthermore, further analysis of isolated CD11b⁺/F4/80⁺ and CD11b⁺/F4/80[−] cells from control and surgically treated tails showed increased VEGF-C expression and a trend for augmented VEGF-D expression in the CD11b⁺/F4/80⁺ macrophages only.

To directly study the biological effects of VEGF-C in lymphedema, we decided to use K14-VEGF-C mice, which chronically express VEGF-C in the skin, mimicking the continuous production of VEGF-C in lymphedema. Surprisingly, however, K14-VEGF-C mice exhibited a significantly exacerbated edema and increased blood vascular leakage after lymphedema surgery, without major differences in the vascular density, but with an increased infiltration of macrophages.

With regard to the potential molecular mechanisms by which VEGF-C overexpression promoted vascular leakage, it has previously been found that fully mature VEGF-C can induce vascular leakage in the skin and mucous membranes via activation of VEGFR2 (Proulx et al., 2013a; Saaristo et al., 2002a, 2002b) and that adenoviral delivery of VEGF-C induced enlargement of blood vessels in a porcine secondary lymphedema model (Visuri et al., 2015).

On the other hand, K14-sVEGFR3-Ig mice express a soluble form of the extracellular domain of VEGFR3 in the

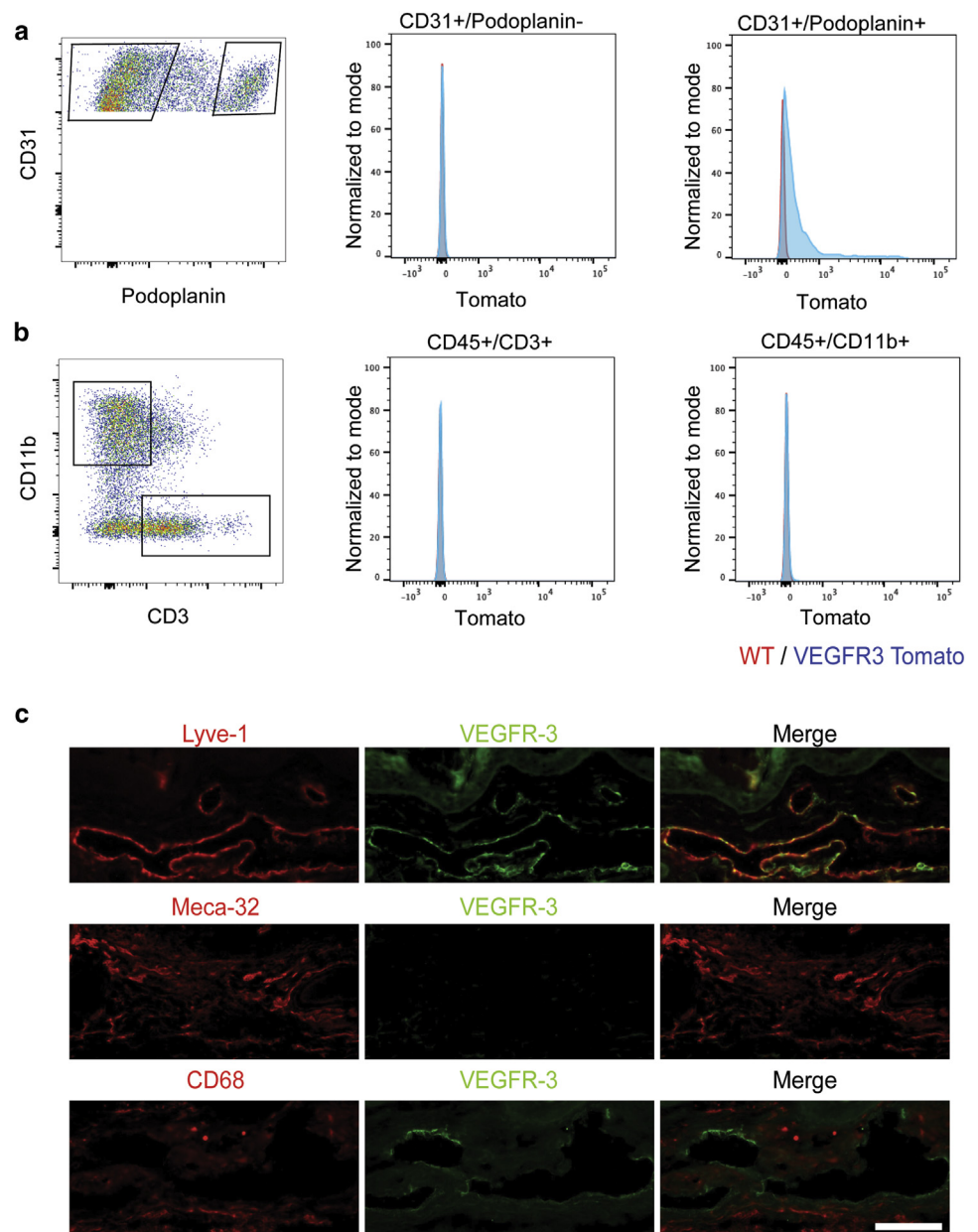
skin, scavenging its ligands VEGF-C and VEGF-D. In these mice, lymphangiogenesis is impaired, whereas the blood vasculature remains functional (Makinen et al., 2001). Our findings that lymphedema development was reduced in K14-sVEGFR3-Ig mice and that there was a reduction of blood vascularity and diminished vascular leakage, further supports the hypothesis that high levels of VEGF-C indeed promote vascular leakage and aggravate lymphedema. This conclusion is supported by our previous findings in an experimental, multistep chemical skin carcinogenesis model, where K14-sVEGFR3-Ig mice had a reduced angiogenesis and vascular leakage (Alitalo et al., 2013).

Although VEGF-D serum levels were not found to be increased after surgery in our mouse tail lymphedema model, the role of locally increased VEGF-D expression should still be considered, because of the increased affinity of the proteolytically processed form toward VEGFR2 (Rissanen et al., 2003; Stacker et al., 1999). In a porcine model of lymphedema, adenoviral delivery of VEGF-D resulted in seroma formation due to the induction of blood vascular permeability (Lahtenvuori et al., 2011), and VEGF-D levels were increased systemically in primary lymphedema patients (Fink et al., 2004). These results indicate a potential role of VEGF-D in edema development, which cannot be excluded in our model because VEGF-D is also scavenged by the soluble form of VEGFR3.

Previous work has reported that exogenously administered VEGF-C can mitigate lymphedema development. Yoon et al. (2003) reported that application of a naked VEGF-C plasmid in nude mice ameliorated lymphedema, but later work suggested that lymphedema was actually self-resolving in those mice because of the absence of CD4⁺ cells (Zampell et al., 2012a, 2012b). Local application of VEGF-C in a mouse tail lymphedema model abrogated tissue damage but led to only a marginal (0.5–1.0% of volume change) decrease of edema (Jin da et al., 2009), indicating that the exact role of exogenously applied VEGF-C still remains unclear.

Despite the promising results of VEGF-C treatment in promoting lymphatic vessel growth, prolonging lymph node survival, and architecture maintenance after lymph node

Figure 6. VEGFR3 expression in mouse tail skin is restricted to lymphatic endothelial cells. VEGFR3 expression was examined 2 weeks after surgery by flow cytometry analysis using VEGFR3-tdTomato transgenic reporter mice and by immunofluorescence co-staining of tissue sections. (a) TdTomato fluorescence was detected in CD31⁺/podoplanin⁺ lymphatic endothelial cells but not in CD31⁺/podoplanin⁻ blood vascular endothelial cells. (b) TdTomato fluorescence was not detected in CD3⁺ T cells and CD11b⁺ myeloid cells. Control mice were VEGFR3-Cre littermates (n = 3). (c) Double immunofluorescence staining of mouse tail skin 2 weeks after surgery for VEGFR3 (green) and LYVE-1, MECA-32, or CD68 (red) showed strong VEGFR3 expression in lymphatic endothelium but not on blood vessels or CD68⁺ macrophages (n = 4). Scale bar = 200 μ m. VEGFR, vascular endothelial growth factor receptor; WT, wild type.



transplantation in lymphedema models (Lahtenvuo et al., 2011; Tammela et al., 2007), a recent study did not report beneficial effects of adenoviral VEGF-C delivery on edema reduction in a porcine secondary lymphedema model (Visuri et al., 2015). Thus, there are concerns that the blood vascular adverse effects, which might largely be mediated via activation of VEGFR2, could counteract the potential activation of lymphatic vessel function. In this regard, our findings that VEGFR3 expression was restricted to lymphatic vessels during lymphedema development suggest that future therapeutic approaches might consider the use of the mutated form of VEGF-C, named VEGF-C156S, that selectively activates VEGFR3 but not VEGFR2 (Joukov et al., 1998; Saaristo et al., 2002b).

We and others have recently found that lymphatic vessel enlargement with lymphatic endothelial cell proliferation is a

characteristic feature of surgically induced lymphedema and that the dilated lymphatic vessels exhibit impaired drainage capacity (Gousopoulos et al., 2016b; Rutkowski et al., 2006). In this regard, our lymphatic drainage assay, showing decreased lymphatic transport capacity in the K14-VEGF-C mice, provides corroborating evidence that the exacerbated edema further impairs lymphatic function. Moreover, there is experimental evidence that high levels of VEGF-C may disrupt and compromise the lymphatic endothelial barrier in mice, thereby increasing lymphatic vascular permeability (Tacconi et al., 2015). Thus, VEGF-C might not only induce leakage of blood vessels but might also induce hyperpermeable, functionally compromised lymphatic vessels, thereby further contributing to the pathogenesis of lymphedema.

Lymphedema represents a multistep disease with complex pathology, where the infiltration of macrophages and local

production of VEGF-C may represent the beginning of a vicious circle leading to further increased edema formation. Therefore, the interaction between VEGF-C and the immune infiltrate, the role of the different immune components, the exact molecular and cellular mechanisms by which VEGF-C regulates vascular permeability, and the normalization of blood vascular leakage as a strategy to control disease progression represent promising fields for future translational research.

MATERIALS AND METHODS

Experimental tail model of lymphedema

Lymphedema was surgically induced in the mouse tail as previously described (Gousopoulos et al., 2016a). Female transgenic mice (on the FVB background) and their female wild-type littermates had surgery at the age of 8–12 weeks. Briefly, a 2- to 3-mm circumferential portion of skin was removed 2 cm distal to the tail base. Subsequently, the deep collecting lymphatic vessels were identified and microsurgically excised, with the lateral tail veins maintained intact. All animal experiments were approved by the Kantonales Veterinäramt Zürich (license number 225/2013).

Tail volume measurements and histology

Tail volume evaluation was performed weekly, using a digital caliper at 1-cm intervals distally to the surgical excision margin. Tail volumes were calculated using the truncated cone formula (Gousopoulos et al., 2016a).

Immunofluorescence stains were performed on 7- μ m-thick cryosections of tail skin embedded in optimal cutting temperature compound (Sakura Finetec, Zoeterwoude, The Netherlands) as previously reported (Gousopoulos et al., 2016). A detailed description of the antibodies used is provided in the [Supplementary Materials and Methods](#) online.

Flow cytometry

To evaluate the different immune populations of the lymphedematous tails, flow cytometry analyses were performed on single cell suspensions obtained from tail skin, as previously described (Gousopoulos et al., 2016b). The lymphedematous skin was stripped off the tail and minced, followed by digestion in a mixture of collagenase II (Sigma, St. Louis, MO) and DNase (Roche, Basel, Switzerland) in RPMI medium (Gibco, Thermo Fisher Scientific, Waltham, MA). The single cell suspension was passed through both 70 μ m and 40 μ m cell strainers and resuspended in FACS buffer. A detailed description of the antibodies used is provided in the [Supplemental Materials and Methods](#) section.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2017.04.033>.

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